Aberrant N-glycosylation in eukaryotic expression system: how the problem is solved?

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Currently several protein expression systems are commonly used in research and industry for expression of variety recombinant proteins. Plant transient expression platform with eukaryotic post-translational modification (PTM) machinery is most promising technology for the production of vaccine antigens, therapeutic proteins, antibodies and industrial enzymes and offers superior efficiency, scalability, safety, and lower cost over other expression systems. However, due to aberrant N-glycosylation of target proteins of interest, this expression system may have significant limitations for those proteins that not carrying N-linked glycans in the native hosts. Bacterial proteins and malaria antigens, and some mammalian proteinsare not glycosylated in their native host, butpossess potential N-glycoslated sites, and therefore, are aberrantly glycosylated when produced in any eukaryotic expression system including plants. The production of these proteins in a eukaryotic system with proper folding is the most challenge task and requires more flexible approaches to preserve their native sequence and biological function. This review article discusses the development of novel molecular biotechnology strategies, which can be used for the production of functionally active recombinant proteins (vaccines, therapeutic proteins, industrial enzymes, native additives etc.) in plants in non-glycosylated form by co-expression with bacterial deglycosylating enzymes, PNGase F and Endo H, and application of strategies for the production of important vaccine antigens.

Keywords: *In vivo deglycosylation, PNGase F, Endo H, vaccines, TB vaccine, Pfs48/45, PA83, anthrax vaccine.*

INTRODUCTION

A number of recent studies demonstrate that N-glycosylation PTM is a critical for the correct folding, and therefore, functional activity, immunogenicity and stability of many proteins produced in different expression systems is dependent on their glycosylation status (Gomord et al., 2010; Mamedov et al., 2012). Mammalian proteins with potential N-glycosylation sites are efficiently glycosylated when expressed in these eukaryotic expression systems. However, the ability of the eukaryotic expression systems to glycosylate proteins may be not desirable for some proteins. If target proteins do not carry N-linked glycans in the native host, but contain potential N-linked glycosylation sites, they can be aberrantly glycosylated in any eukaryotic expression system, thus, potentially impairing the native folding and biological activity. Some eukaryotic proteins such as malaria antigens, for example, Pfs48/45 protein of *Plasmodium falciparum* and also antigensofa wide range of bacteria, for example, Protective antigen (PA)of *Bacillus anthracis* are not N-glycosylated in native host but contain potential N-glycosylated sites. Another example is A subunit of human factor XIII, which isproduced by native hosts as complex post-translationally modified proteins, however do not carry N-linked glycans, but contain potential N-linked glycosylation sites (Ashcroft et al., 2000; Schwartz et al., 1971). Therefore, these proteins are aberrantly glycosylated during expression in yeast,

mammalian, or plant systems. It has been shown that aberrant N-glycosylation makes a lot of problems for many therapeutic applications. AberrantN-glycosylation is often observed in proteins of cancer cells (Nita-Lazar et al., 2009), cell surface receptors, integrins and cadherins, which was associated with changes in carcinoma progression and metastases, indicating significant changes in the behavior of these proteins (Guo etal., 2002; Partridge et al., 2004). Taking together, all these findings indicate that attachment of carbohydrates to protein backbone strongly affects physico-chemical properties of a protein, and therefore alter its essential biological properties such as the specific activity, immunogenicity, stability etc. At this point, eukaryotic expression systems may not be desirable expression system for those proteins (for example, bacterial proteins or malaria antigens) that do not require N-linked glycosylation. Therefore, it is very important to develop strategies for production of non-glycosylatedforms of target proteins in eukaryotic expression system while preserving their native conformation and biological activity. Previously, there were some efforts of blocking of N-glycosylation of proteins in plants. For example, N-glycosylation was blocked with a tunicamycin, a powerful antibiotic against gram-positive bacteria and a specific inhibitor of the enzyme that transfers acetylglucosaminephosphate on to dolichol phosphate, results in a non-uniform expression of proteinsin plants (Frank et al., 2008; Hori and Elbein, 1981). It should be noted that tunicamycinis very toxic, significantly affects protein folding (D'Amico et al., 1992; Sparvoli et al., 2000) and inhibits extracellular secretion of proteins (Faye and Chrispeels, 1989) and also a long-term treatment with tunicamycin has a lethal effect on plants (Gomord et al., 2010). Therefore, this strategy may not be practical and useful for the production of recombinant proteins in the non-glycosylated form. To overcome the problem, recently a robust strategies were developed for production of recombinant proteins in non-glycosylated form in plants by co-expressing bacterial peptide-N-glycosidase F (PNGase F) or Endo-β-N-acetylglucosaminidase (EC3.2.1.96, Endo H) with target proteins. PNGase F is a 34.8-kDa enzyme, secreted by a Gram-negative bacterium *Flavobacterium meningosepticum* (Plummer et al., 1984; Tarentino et al., 1990) and cleaves a bond between the innermost GlcNAc and asparagine residues of high-mannose, hybrid and complex oligosaccharides. It should be noted that, deglycosylation by PNGase F (*in vivo or in vitro* deglycosylation) does cause an amino acid change in the deglycosylated protein at the glycosylationsite (N-X-S/T) due to the deamidation of asparagine to aspartate (HaÈgglund et al., 2004; Mamedov et al., 2012). Endo H enzyme catalyzes cleavage between two N-Acetyl-D-glucosamine (GlcNAc) residues of the chitobiose core of N-linked glycans with great efficiency in the diacetylchitobiose core of *N*-linked glycans, leaving a single N-Acetyl-D-glucosamine residue without the concomitant deamidation ofasparagine (Muramatsu 1971, Maley et al., 1989; Tarentino and Maley,1976). Since the plant expression system becomes the most promising platform for expression of recombinant proteins, therefore, considerable efforts have been made to produce functionally active and stable malaria (Pfs48/45 based) and anthrax (PA83 based) vaccine candidate antigens using the plant transit expression system. However, the Pfs48/45 protein, which was produced earlier in plants at Fraunhofer USA Center for Molecular Biotechnology (FhCMB), as a aberrantly glycosylated form had very low transmission blocking (TB) activity (see Mamedov et al., 2012; Mamedov and Yusivov, 2013). Similarly, PA83 proteinwithnine potential N-glycosylation sites is aberrantlyglycosylated in plants, and thereforethis molecule (Chichester et al, 2007; 2013) had no toxin neutralizing activity and could not form LeTx *in vitro*. Developing *in vivo* deglycosylation strategies enabled production of functional active and stable PA83 and Pfs48/45 proteins in plants (Mamedov et al., 2012; Mamedov et al., 2017).

Production of non-glycosylated Pfs48/45, a malaria vaccine candidate, and PA83, an anthrax vaccine candidate, in *N. benthamiana* plants by co expression with bacterial de-glycosylating enzymes; aberrant glycosylated proteins versus non-glycosylated counterparts

Although a number of expression systems available, however these protein expression platforms may not be suitable for expression of

particular proteins. *Plasmodium falciparum* is one of the deadliest parasite in human history and therefore, developing safe, low-cost and highly efficient malaria vaccine with long-term stability is urgently needed. *Plasmodium falciparum* is a unicellular eukaryoticorganism, which may have complex post-translationally modified proteins such as Pfs48/45; Psf48/45 is one of the leading candidates for transmission blocking (TB) vaccine development and plays a key role in parasite fertilization. Pfs48⁄45 do not carry N-linked glycans (Milek et al., 1998) but contain potential N-linked glycosylation sites that can be aberrantly glycosylated during expression in eukaryotic expression systems. Although expression of full-length Pfs48/45 has been tried in several recombinant protein expression systems, but the resulting proteins have not induced TB antibodies in mice. A challenge to developing a vaccine based on Pfs48/45 is the production of correctly folded recombinant protein. Pfs48/45 is a complex cysteine rich (16 cysteines involved in disulfide bond formation) membrane anchored protein with seven predicted N-linked glycosylation sites. Proper folding of many cysteine-rich proteins, including Pfs48/45, depends on correct formation of disulfide bridges. In addition, *Plasmodium* parasites are one of the few eukaryotes that lack the N-linked glycosylation machinery, and however, many *Plasmodium* proteins contain multiple potential glycosylation sites, which are aberrantly glycosylated when expressed in any of the available eukaryotic hosts. Since mammalian expression system is extremely expensive, and plus there is always risk with pathogen contamination, this system may not be suitable expression system for production of a safe and cost effective malaria TB vaccine. In addition, since Pfs48/45 is a complex cysteine rich (16 cysteines involved in disulfide bond formation) protein, bacterial expression system may not be capable for the production of properly folded (disulfide-bonded**)** full lengthPfs48/45 protein (Outchkourov et al., 2008). Similarly, all bacterial antigens that are leading candidates for vaccine development may have several potential N-glycosylation sites and can be aberrantly glycosylated in any eukaryotic expression system, including plants. Therefore, the plant expression system may not be a suitable platform for the production of these bacterial antigens in plant cells.

All the drawbacks discussed above for the production of important vaccines against malaria and bacterial antigens have led to the development of new strategies for the production of non-N-glycosylated recombinant proteins, including Pfs48/45 or PA83 in the plant system. Thus, for the expression of various proteins with different post-translational modification status, more flexible approaches are needed to preserve their natural sequence and biological function. At this point, we recently developed a strategy a strategy of enzymatic deglycosylation of proteins *in vivo* by co-introducing bacterial PNGase F via agroinfiltration followed by transient expression in plant (Mamedov et al., 2012). Pfs48/45 protein produced by PNGase F *in vivo* deglycosylation strategy was recognized by monoclonal antibodies I, III and V raised against various epitopes (I, III and V) of native Pfs48⁄45 of *Plasmodium falciparum*, significantly stronger compared to that of the glycosylated form of plant-produced Pfs48⁄45 (Mamedov et al., 2012). Similarly, deglycosylatedPA83 protein, leading vaccine candidate against anthrax, produced by PNGase F *in vivo*deglycosylation strategy was functional active and could form LeTx*in vitro* (Mamedov et al., 2016).In addition, when purified plant produced deglycosylated PA83 was assessed for stability, it appeared to be more stable than the glycosylated counterpart (Mamedov et al., 2016).Though PNGase F removes oligosaccharides from glycosylated proteins, in so doing it causes an amino acid change due to the deamidation of asparagine to aspartate in the N-X-S/T site. Therefore, we recently reported on the development of a more advanced strategy for the production of recombinant proteins in plants in non-deglycosylated form (Mamedov et al., 2017). This strategy represents of enzymatic deglycosylation of proteins *in vivo* by co-introducing bacterial Endo H, which catalyzes cleavage between two N-Acetyl-D-glucosamine residues of the chitobiose core of *N*-linked glycans, leaving a single N-Acetyl-D-glucosamine residue without the concomitant deamidation of asparagine (Mamedov et al., 2017). Endo H deglycosylation approach was

successfully applied to generate non-glycosylated recombinant deglycosylated PA of *B. anthracis* and Pfs48/45 proteins of P. *falciparum*. It should be noted that there have been many years of efforts to develop a vaccine against the anthrax based on PA of *B. anthracis* (Chichester et al, 2007, 2013). However, as mentioned above the plant produced PA83 protein was not functional active and could not form LeTx *in vitro*. In addition, it has recently been demonstrated that (Mamedov et al., 2017) the glycosylated plant produced PA83, previously developed as an anthrax vaccine candidate (Chichester et al, 2007, 2013), is very unstable and degrades by more than 90% at 37ºC for 8 hours. At the same condition, Endo H deglycosylated PA83 showed super stability compare with glycosylated PA83 and degraded less that 25% (Mamedov et al., 2017). Endo H deglycosylated PA83 showed a better stability compared to PNGase F deglycosylated PA83 and superior stability compared to glycosylated PA83. These results demonstrate that Endo H deglycosylated PA83 is expected to be the most advanced candidate for a new generation of PA83 vaccine against anthrax in terms of cost, safety, stability and immunogenicity. Endo H deglycosylation strategy was also successfully applied for the production of plant produced Pfs48/45 protein. It was demonstrated that, in contrast to the glycosylated form, plant produced *in vivo* Endo H deglycosylated Pfs48/45 was recognized by conformational specific Pfs48/45 monoclonalantibody, a known TB antibody, in a manner similar to its PNGase F deglycosylated counterpart (Mamedov et al., 2017). It should be noted that the IIC5B-10-1 mAbs is known to target antigens of TB immunity on gametes of *P. falciparum*. Notable, Pfs48/45 was also previously expressed in *N. benthamiana* plant at FhCMBusing a transient expression system, but the TB activity of this plant derived vaccine candidate was very low (Mamedov&Yusibov, 2013). Thus, plant produced Endo H *in vivo* deglycosylated Pfs48/45 antigen has a potential for the development of a Pfs48/45-based TB malaria vaccine. All these findings demonstrate that aberrant N-glycosylation PTMs of recombinant proteins produced in plant (eukaryotic) expression systems lead to significantly reduced functionality, immunogenicity and stability and impaired functional activity. Collectively, all of the above facts confirm that the Endo H deglycosylation strategy provides great opportunities for the production of functional active, valuable vaccine antigens, therapeutic proteins, antibodies and recombinant enzymes for therapeutic use and industrial application.

CONCLUSIONS

In summary, due to aberrant N-glycosylation, an eukaryotic expression system, including plant, had a significant limitation for the production of a wide range of proteins, including bacterial proteins (important bacterial vaccine antigens, industrial enzymes, native additives etc.), malaria antigens etc. In other words, the *in vivo* deglycosylation strategy has expanded the utility and use of the eukaryotic expression system including plants for the production of a wide range of pharmaceuticals. The *in vivo* enzymatic deglycosylation strategy was successfully developed and applied to solve the problem, associated with aberrant N-glycosylation. In fact, this strategy allowed the production of functionally active, immunogenic PA83 and Pfs48/45 proteins with long-term stability in the plant system at reduced costs. The *in vivo* deglycosylation efficiency of plant produced Endo H greater than that of plant produced PNGase F (Mamedov et al., 2017). In addition, Endo H deglycosylation had more positive impact on the stability and potency of co-expressed recombinant proteins compare to that of PNGase F deglycosylation. Thus, Endo H *in vivo* deglycosylation approach are highly expected to have potential applications in molecular farming, in the pharmaceuticals field to produce subunit vaccines, therapeutic proteins, and antibodies in the deglycosylated forms. The strategy can be also important for the production of industrial enzymes, especially bacterially originated enzymes, in eukaryotic expression system for increasing bioenergy, and biofuel yield, as well as improving food quality, especially by producing native additives. In conclusion, recent studies (Mamedov et al., 2012; Mamedov et al., 2016; Mamedov et al., 2017) collectively demonstrate

that enzymatic deglycosylation of target proteins *in vivo* has the potential to become a robust strategy for the production of non-glycosylated proteins in plants.

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Eukariot ekspressiya sistemində aberrant N-qlikozillləşmə: problem necə həll olunur?

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Hazırda müxtəlif recombinant zülalların tədqiqatlarda və sənaye miqyasında ekspressiyası üçün adətən zülalların bir neçə ekspressiya sistemi istifadə olunur.Eukariotların post-translyasion modifikasiya maşınını istifadə etməklə bitkilərdə ekspressiya üçün olan platform vaksin antigenlərin, terapevtik zülalların, anticisim və sənaye fermentlərinin istehsalı üçün ən perspektivli texnologiyadır və digər ekspressiya sistemləri ilə müqayisədə yüksək effektivliyi,miqyaslılığı, təhlükəsizliyi və daha aşağı qiyməti təmin edir. Lakin, maraq kəsb edən məqsədli zülalların aberrant N-qlikozilləşməsi üzündən bu ekspressiya sistemi təbii sahibləri N-birləşmiş qlikonlar daşımayan zülallar üçün bu ekspressiya sistemi xeyli məhduduyyətlərə malik ola bilər. Bakteriya zülalları və malyariyanın antigeni, həmçinin, məməlilərin bəzi zülalları özünün təbii sahiblərində qlikozilləşmirlə, amma potensial N-qlikozilləşmə saytlarına malikdirlər və uyğun olaraq bitkilərdə daxil olmaqla istənilən eukariot ekspressiya sistemində sintez zamanı aberrant qlikozilləşmişdir. Eukariot sistemlərdə bu zülalların düzgün yığılma ilə istehsalı daha mürəkkəb məsələdir və onların nativ ardıcıllığının və bioloji funksiyaların saxlanması üçün daha incə yanaşmalar tələb edir. Bu icmal məqalədə funksional aktiv recombinant zülalların (vaksin, terapevtik zülallar, sənaye fermentlər və native əlavələr) bitkilərdə qlikozilləşməmiş formada bacterial deqlikozilləşmiş fermentlər, PNGase F və Endo H ilə koekspressiyası yolu ilə istehsalı üçün istifadə oluna bilən molekulyar biotexnologiyanın yeni strategiyaların işlənib hazırlanması və vacib vaksin antigenlərin istehsalı üçün strategiyaların tətbiqi müzakirə olunur.

Açar sözlər: *In vivo deqlikozilləşmə, PNGase F, Endo H, vaksinlər, TB vaksin, Pfs48/45, PA83, qarayara vaksini*